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Comparison of cellular and humoral immunoassays for the assessment of summer eczema in horses

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Abstract

The objective of this study was to compare and analyze three common diagnostic methods for summer eczema (SE) in horses, an allergic dermatitis caused by bites of *Culicoides* spp. Nine horses with a medical history of SE and nine control animals were intradermally challenged with whole body extracts (WBE) and the saliva of a native (*C. nubeculosus*) and exotic (*C. sonorensis*) *Culicoides* species. Blood and serum samples of the horses were examined for basophil reactivity by a histamine release test (HRT) and for *Culicoides*-specific serum immunoglobulin E (IgE) and G (IgG) by enzyme-linked immunosorbent assay (ELISA). The results of intradermal testing (IDT) at 30 min (immediate reactivity) and 4 h (late-phase reactivity) post challenge with most insect preparations revealed significant differences between horses with and without SE. Overall, the HRT showed the most accurate results with a sensitivity of 1.00 for all *Culicoides* preparations and specificities of 0.78 (WBE) and 1.00 (saliva). By contrast, delayed reactions of the IDT (24 h), and levels of *Culicoides*-specific IgE and IgG in the native serum showed little or no distinction between allergic and non-allergic horses. However, the use of purified serum IgE and IgG indicated the possibility for elevated titers of insect-specific serum immunoglobulins in horses with SE. The IDT and HRT did not reveal obvious differences in onset and intensity of positive reactions for the native verses exotic *Culicoides* species, whereas the ELISA showed slightly higher numbers of positive reactions for serum IgG with the indigenous species. Saliva, as compared to WBE, was found to have improved sensitivity and/or specificity for the HRT and for the late-phase immune reactions as measured by the IDT. Overall, the results indicate that allergy tests utilizing effector cells (mast cells, basophils) are more accurate in diagnosing SE in horses than serological analysis by ELISA.

Keywords: *Culicoides* spp; Equine; Summer eczema; Skin testing; Histamine release test; Serological testing

1. Introduction

Summer eczema (SE), also known as sweet itch or insect bite hypersensitivity, is a seasonal recurrent allergic dermatitis that affects a substantial number of horses worldwide (Anderson et al., 1988; Braverman,

Abbreviations: IDT, intradermal test; HRT, histamine release test; ELISA, enzyme-linked immunosorbent assay; SE, summer eczema; SE+ horses, horses with summer eczema; WBE, whole body extract; Ig, immunoglobulin.

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1988; Halldorsdottir and Larsen, 1991; Littlewood, 1998). The disease has been reported for various breeds including Icelandic horses, Quarter Horses, Warmblood Horses and different pony breeds. Summer eczema is associated with severe pruritus, local alopecia and self excoriation, often followed by secondary bacterial or fungal infections. Different species of hematophagous insects, in particular midges of the genus *Culicoides*, have been shown to induce SE (Quinn et al., 1983; Fadok and Greiner, 1990). It is assumed that components of the insect saliva initiate an immediate (type I) hypersensitivity reaction in atopic horses that is mediated by IgE and possibly isotypes of IgG (Wilson et al., 2001; Hellberg et al., 2006; Wagner et al., 2006). Additionally, evidence for a delayed reaction (type IV hypersensitivity) induced by allergen specific T-cells was found in some SE affected (SE+) horses (McKelvie et al., 1999; Ferroglio et al., 2006). The diagnosis of the allergy primarily relies upon the physical examination of the clinical signs and the exclusion of pathogen-induced disorders which may lead to similar clinical disease. In addition, *in vivo* and *in vitro* tests are used to confirm the diagnosis or to evaluate the allergic condition in horses in the absence of clinical signs.

Currently, three different test systems are available for the diagnosis of SE in horses. Intradermal testing (IDT) with *Culicoides* preparations is widely used to determine hypersensitivity by demonstrating local mast cell activation (Halldorsdottir et al., 1989; Fadok and Greiner, 1990; Anderson et al., 1993; Kolm-Stark and Wagner, 2002; Ferroglio et al., 2006). Evaluation of the skin test site at 30 min and at 1 to 6 h determines the immediate and late-phase reaction of an IgE-dependent type I hypersensitivity, respectively. Evaluation at 24 h or later after the injection determines a delayed reaction due to type IV hypersensitivity. In addition, different histamine release tests (HRT) have been developed that utilize equine basophilic granulocytes (basophils) obtained from blood samples (Kaul, 1998; Marti et al., 1999; Baselgia et al., 2006). Similar to mast cells, basophils carry allergen specific IgE and possibly IgG which induce the release of various mediators (e.g., histamine, leukotrienes) from the cells upon cross-linkage by suitable allergens. The amount of the released mediators after *in vitro* stimulation of the cells with *Culicoides* preparations is assumed to correlate with the allergic status of the horse, discriminating between animals with and without SE. Recently, reagents specific for equine IgE antibodies have been produced (Kalina et al., 2003; Wagner et al., 2003; Wilson et al., 2006) which enabled the development of serological tests, in particular the ELISA, for the diagnosis of equine hypersensitivities. In contrast to IDT and HRT which record the cellular reaction, the ELISA determines the humoral response by measuring equine serum antibodies specific for allergens. Although an ELISA system for hypersensitivities to the bite of *Culicoides* spp. midges has not yet been described in the literature, the measurement of serum antibodies with this system has been used previously to determine equine hypersensitivities to different allergens including grass and grain mill dust, as well as the bites of mosquitoes and horse flies (Lorch et al., 2001; Kalina et al., 2003).

Diagnostic tests to determine SE in horses are offered by several companies and institutes. Individual methods have been performed on large numbers of horses and are well classified. However, the use of different and often non-standardized allergen preparations severely impedes correlation of the test results. In addition, climate and habitat factors can significantly influence the allergic status of horses (Grevenhof et al., 2007) and may affect the reproducibility of test results obtained from horses in different environments. In the present study, IDT, HRT and ELISA detection methods were carried out in comparison with standardized allergen preparations in 18 Icelandic horses (nine affected by SE, nine control animals) that were held under identical conditions. *Culicoides* whole body extracts (WBE) from the Northern European species *Culicoides nubeculosus* and the North American species *Culicoides sonorensis* were used for the testing. *C. nubeculosus* is known to induce SE in horses in Northern Germany (Kaul, 1998) whereas *C. sonorensis* was shown to be involved in the pathogenesis of the allergy in southeastern USA (Fadok and Greiner, 1990). Additionally, collected midge saliva from both species, which is considered to be the primary source of allergens, was examined for its ability to improve the test systems. The results obtained for the individual methods were analyzed and their reliability for the diagnosis of SE in horses evaluated.

2. Materials and methods

2.1. Horses

A total of 18 Icelandic horses, ages 5–17 years (average 11 years), were included in the study. Horses were maintained together in a geographic area endemic for *C. nubeculosus* in Northern Germany and exposed to insect bites under natural conditions for several seasons. Nine horses, ages 12–17 years (average 13 years), showed clinical signs of SE for at least 7 years with hair loss, crusting and open wounds in the mane, withers and base of the tail, and occasionally along the ventral midline and on the head. The intensity and localization of clinical signs varied between individuals annually. Nine horses with no clinical evidence of SE that were held together with the SE+ horses in the same environment for at least 5 years were used as negative control animals. Three of the control horses were imported from Iceland 12 years ago. All horses were vaccinated and treated regularly for intestinal parasites. No anti-inflammatory drugs were given for at least 3 months prior to the testing.

2.2. Blood and serum sampling

Blood samples were collected on the day of IDT in Vacutainer® K2E tubes (Becton Dickinson, Heidelberg, Germany) for whole blood and Vacutainer® CAT tubes (Becton Dickinson) for sera, which was obtained after centrifugation at $1000 \times g$ for 10 min and stored at $-20\,^{\circ}$C. Serum-IgE and -IgG of five SE+ horses and five control animals were purified according to a modified method of Wagner et al. (2003). Briefly, 1 ml of each serum sample was diluted 1:25 in phosphate buffered saline (PBS) and applied to 1 ml protein G sepharose columns (GE Healthcare, Uppsala, Sweden) to bind IgG. The flow-through was subsequently applied to 1 ml cyanbromide-activated sepharose columns (GE Healthcare) coupled with a monoclonal antibody specific for binding equine IgE (Wagner et al., 2003). Following two washes with 10 ml PBS, purified serum-IgG and IgE were eluted from the columns using 3 ml 0.1 M glycine (pH 3.0), and neutralized in 1.5 ml 1 M Tris(hydroxymethyl)aminomethane, pH 7.0. Eluates were concentrated to a final volume of 100 μl using ultrafiltration units with a 100 kDa cut-off (Vivaspin 500, Vivascience, Hannover, Germany). The elution buffer in the concentrate was exchanged by subsequent addition of 2 ml PBS and centrifugation back to a volume of 100 μl. Samples were analyzed for purity on 7.5% non-denaturing SDS gels after staining with Coomassie Brilliant Blue (Sigma–Aldrich, Taufkirchen, Germany). The Bicinchoninic Acid (BCA) Protein Assay (Perbio Science, Rockford, IL) was used to determine the protein concentration as per the manufacturer's instructions.

2.3. Insects

C. nubeculosus and *C. sonorensis* were reared at the Institute for Animal Health in Pirbright, UK, according to the method of Boorman (1974). Three-day-old unfed insects, frozen at $-70\,^{\circ}$C, were used for the preparation of WBE. Whole midges were ground in chilled extraction buffer (10 mM ammonium hydrogen carbonate, pH 7.0). The suspension was centrifuged at $3000 \times g$ at 4 °C for 15 min to pellet insoluble material. The supernatant was filtered (0.22 μm, Renner, Darmstadt, Germany) and loaded onto a PD 10 desalting column (Amersham Bioscience, Freiburg, Germany) with PBS exchanges of extraction buffer as per the manufacturer's instructions. The BCA assay was used to determine the protein concentration as above, and extract aliquots were stored at $-20\,^{\circ}$C. Saliva collection was carried out as previously described (Langner et al., 2007). Briefly, three to 5-day-old *C. nubeculosus* or *C. sonorensis* were placed repeatedly onto a saliva collection system that was modified from an artificial membrane feeding device for *Culicoides* spp. (Mellor, 1971; Venter et al., 1991). Sucrose-soaked filters (Durapore™, Millipore, Eschborn, Germany) on top of the device covered with Parafilm (American National Can, Greenwich, CT) served as collection membranes. Salivary proteins were eluted from the filters by shaking over night at 4 °C in PBS and concentrated to a final volume of 1 ml using ultrafiltration units (Vivaspin 20, cut-off 3 kDa, Vivascience). Protein concentrations of the eluates were determined as above and stored at 4 °C until use.

2.4. Intradermal test

The IDT was performed in late fall of 2005 in the absence of clinical SE in the 18 horses. A 30 cm × 15 cm area in the dorsolateral cervical region was closely clipped and disinfected with 70% ethanol. The individual injection sites were indicated with a permanent marker. Prior to the study, a 10-fold dilution series of WBE and saliva ranging from 50 to 0.005 μg/ml were tested in two SE+ horses and two control horses to determine the optimal concentrations for the IDT. Histamine (Sigma–Aldrich) was dissolved at a concentration of 0.2 mg/ml in PBS and served as positive control and PBS was used for the negative control. A volume of 0.05 ml of each preparation was injected intradermally using 1 cc syringes and 28 gauge needles. Skin reactions were assessed at 30 min, 4 h and 24 h postinjection (p.i.) by measuring the two greatest diameters of the resulting wheal and calculating the average. Evaluation was done according to the method described by Kolm-Stark and Wagner (2002). A (1+) or (2+) reaction designation was given to wheals one-quarter or one-half size of the sum of the negative and positive control. A (3+) reaction was half of the sum of the (2+) reaction and the positive control. A reaction equal to or greater than the reaction provoked by histamine was designated as (4+). Reactions equal to or greater than (2+) were considered positive.

2.5. Histamine release test

The HRT was performed according to a modified method of Kaul (1998). Briefly, red and white blood cells obtained from Na–EDTA coagulation inhibited blood were washed twice with PBS. *Culicoides* WBE and saliva were prepared in a 10-fold dilution series in releasing buffer (110 mM sodium chloride, 40 mM sodium hydroxide, 25 mM piperazine *N*,*N*′bis[2-ethanesulfonic]acid, 5 mM potassium chloride, 2 mM potassium chloride, 2 mM magnesium chloride, pH 7.4) ranging from 5 to 0.00005 μg/ml. Samples were incubated with the washed blood cells at 37 °C for 1 h and then held on ice for 20 min. The supernatant containing the released histamine was collected from each sample after centrifugation and stored at −20 °C until further processing. The incubation of releasing buffer with washed blood cells served as a negative control (spontaneous release). Antibodies specific for equine IgE (Wagner et al., 2003) and IgG (Jackson Immuno Research Laboratories, West Grove, PA) were used as positive controls. The maximal histamine content was obtained by boiling the blood cells for 10 min in a water bath (maximal release). Histamine concentrations were determined using a competitive RIA (LDN Nordhorn, Germany) as per the manufacturer's instructions. The amount of histamine obtained for the maximal release was set as 100%. Histamine release equal to or greater than 10% of the maximal release were considered as positive. The horses were scored as (1+) to (4+) according to dilution factor of the allergen preparation still capable of inducing a positive reaction. To ensure specificity of the HRT, only horses that were responsive with the positive controls, but had spontaneous releases less than 10% of the maximal release, were included in the evaluation.

2.6. ELISA

Culicoides extracts and salivary proteins at a concentration of 0.4 μg per well in coating buffer (35 mM sodium hydrogen carbonate, 5 mM sodium carbonate, pH 9.6) were used to sensitize 96 well polystyrene plates (Immunlon, Dynatech, Alexandria, VA). The optimal coating concentration was determined prior to the study by titration of the preparations. The plates were incubated overnight at 4 °C, then blocked at RT for 1 h with 0.5% gelatin in washing buffer (145 mM sodium chloride, 75 mM disodium hydrogen phosphate, 25 mM sodium hydrogen phosphate, 0.1% Tween 20, pH 7.2) and rinsed five times with washing buffer. Optimal serum dilutions and antibody concentrations were determined prior to the study (data not shown). Sera of allergic and non-allergic horses were diluted 1:3 in washing buffer for the detection of *Culicoides*-specific IgE and 1:300 for the detection of IgG. Purified serum IgE and IgG were used at concentrations of 30 μg per well. Sera or purified antibodies from non-allergic horses were pooled and diluted as above for negative reference. All samples were plated in duplicate. Plates were incubated at RT for 1 h and washed as above. For the detection of IgE, a murine monoclonal antibody with specificity for equine IgE (anti-IgE 134, Wagner et al., 2003) was added to the wells at a 1:3 dilution of cell culture supernatant and incubated at RT for 3 h. Following a washing step, horseradish peroxidase-labelled goat-anti-mouse antibody, specific for murine IgG and IgM (Jackson Immuno Research Laboratories), was added at a 1:5000 dilution and incubated at RT for 1 h. For the detection of IgG, the plates were incubated at RT for 1 h with horseradish peroxidase-labelled goat-anti-horse antibody specific for equine IgG (Jackson Immuno Research Laboratories). Following a final wash, plates were developed using 15 mM Tetramethylbenzidine and 5 mM hydrogen peroxide in substrate buffer (67 mM disodium hydrogen phosphate, 33 mM citric acid, pH 5.0). After 20 min in the dark, the reaction was stopped by adding 1N sulfuric acid. Plates were read at 450 nm (Synergy HT, Biotek Instruments, Winooski, VT). Results were evaluated according to the method described by Kalina et al. (2003). Briefly, the OD readings of the samples were compared with the serum or antibody pool of the non-allergic horses using the sample to negative (S/N) ratio. A ratio <1.5 was defined as negative, a ratio between 1.5 and 1.99 as borderline, between 2.0 and 4.0 as positive, and >4.0 as highly positive.

2.7. Statistical analysis

The software GraphPad Prism was used for the statistical analysis utilizing contingency tables. The total number of positive and negative reactions (2 × 2 comparisons) of the IDT, HRT and ELISA were performed using Fisher's exact test. The Chi-squared test was used to evaluate the different levels of positive reactions (2 × 4 comparison) obtained for IDT and HRT. Effects were considered significant at $P < 0.05$. Specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) for all test systems were calculated according to the method of Gerstman and Cappucci (1986). Sensitivity was defined as the proportion of SE+ horses with positive test results (true positive), whereas specificity was represented by the proportion of control animals with negative test results (true negative). The PPV described the proportion of SE+ horses with positive test results out of all positive test results (true positive + false positive) and the NPV as the proportion of control horses with negative test results out of all negative test results (true negative + false negative).

3. Results

3.1. Intradermal test

At 30 min post-injection (p.i.), all horses had developed skin reactions at the site of the histamine injection (positive control). The diameter of the wheal had decreased at 4 h p.i. and no response was observed at 24 h p.i. Wheals at the site of saline injection (negative control) were only present at 30 min p.i. in all horses. There was no difference between affected and unaffected horses in the mean response to histamine and saline.

Antigen titration in two SE+ horses and two control animals revealed an optimal range of 5 to 0.05 μg/ml for all *Culicoides* preparations used for the main study (data not shown). At 30 min p.i., the highest WBE and saliva antigen concentrations of 5 μg/ml induced skin reactivity in 72% of SE+ horses (total of responders out of 36 possible reactions [four allergen preparations × nine horses]) and 0% of unaffected horses (Table 1). Both 2 × 2 and 2 × 4 comparisons showed significant differences for the mean number of positive reactions for all preparations. In addition, a small number of SE+ horses reacted to one or more preparations at the next higher dilutions of 0.5 μg/ml (17%) and 0.05 μg/ml (5.5%).

At 4 h p.i., no significant difference was seen in the 2 × 2 comparisons at the highest concentration (5 μg/ml) for all preparations, with positive reactions observed in 94% of SE+ horses and 80% of unaffected horses (Table 2). However, wheal diameters were larger in SE+ horses than in control animals at this concentration, resulting in significant differences in the 2 × 4 comparisons for the *C. sonorensis* WBE and the saliva of both species. Significant differences were seen in both the 2 × 2 and 2 × 4 comparisons between SE+ and controls when horses were challenged with higher dilutions of *C. nubeculosus* WBE and both species of insect saliva.

At 24 h p.i. positive reactions to the 5 μg/ml preparations were observed for 47% of the SE+ horses and 22% of the control horses (Table 3). The next higher dilution of 0.5 μg/ml induced a delayed reactivity in 8%

Table 1
Number of responses to IDT at 30 min after the challenge for SE affected horses ($n = 9$) and control animals ($n = 9$)

Preparation	Concentration (μg/ml)	Skin reaction for horses with SE				Skin reaction for control horses				Significance	
		Negative	2+	3+	4+	Negative	2+	3+	4+	2 × 2[a]	2 × 4[b]
C. nubeculosus WBE[c]	5	2	5	1	1	9	0	0	0	0.002**	0.009**
	0.5	7	1	0	1	9	0	0	0	0.471	0.325
	0.05	8	1	0	0	9	0	0	0	1	1
C. sonorensis WBE	5	2	5	2	0	9	0	0	0	0.002**	0.003**
	0.5	8	1	0	0	9	0	0	0	1	1
	0.05	9	0	0	0	9	0	0	0	ns[d]	ns
C. Nubeculosus saliva	5	2	6	1	0	9	0	0	0	0.002**	0.003**
	0.5	8	1	0	0	9	0	0	0	1	1
	0.05	9	0	0	0	9	0	0	0	ns	ns
C. sonorensis saliva	5	4	4	1	0	9	0	0	0	0.029*	0.031*
	0.5	7	2	0	0	9	0	0	0	0.471	0.471
	0.05	8	1	0	0	9	0	0	0	1	1

[a] Level of significance in 2 × 2 comparisons for negative (<2+) and positive (≥2+) reactions.
[b] Level of significance in 2 × 4 comparisons for negative (<2+) and (2+), (3+) and (4+) positive reactions.
[c] WBE, whole body extract.
[d] ns, no statistical analysis as allergen is constant.
* $p < 0.05$.
** $p < 0.01$.

Table 2
Number of responses to IDT at 4 h after the challenge for SE affected horses ($n = 9$) and control animals ($n = 9$)

Preparation	Concentration (μg/ml)	Skin reaction for horses with SE				Skin reaction for control horses				Significance	
		Negative	2+	3+	4+	Negative	2+	3+	4+	2×2[a]	2×4[b]
C. nubeculosus WBE[c]	5	1	3	4	1	4	4	1	0	0.294	0.231
	0.5	2	5	1	1	8	1	0	0	0.015*	0.041*
	0.05	7	1	1	0	9	0	0	0	0.471	0.471
C. sonorensis WBE	5	0	1	4	4	2	6	1	0	0.471	0.009**
	0.5	2	4	3	0	6	3	0	0	0.153	0.076
	0.05	6	3	0	0	9	0	0	0	0.206	0.206
C. nubeculosus saliva	5	0	1	4	4	0	8	1	0	ns[d]	0.003**
	0.5	1	6	1	1	4	5	0	0	0.294	0.274
	0.05	3	6	0	0	9	0	0	0	0.009**	0.009**
C. sonorensis saliva	5	1	0	7	1	1	7	1	0	ns	0.006**
	0.5	2	4	2	1	4	5	0	0	0.62	0.287
	0.05	3	5	1	0	9	0	0	0	0.009*	0.011*

[a] Level of significance in 2×2 comparisons for negative (<2+) and positive (≥2+) reactions.
[b] Level of significance in 2×4 comparisons for negative (<2+) and (2+), (3+) and (4+) positive reactions.
[c] WBE, whole body extract.
[d] ns, no statistical analysis as allergen is constant.
* $p < 0.05$.
** $p < 0.01$.

of the allergic horses and in 3% of the control animals. Due to the absence of a wheal at the injection site of the histamine control, no 1+ to 4+ scores were calculated for the delayed reactivity. Comparison of the total positive and negative reactions did not reveal significant differences between horses affected by SE and control horses.

3.2. Histamine release test

All SE+ horses and control animals had strong responses to the positive controls indicating the presence of sensitizing IgE and IgG on the surface of basophils. The spontaneous release did not exceed 10% in either of the groups. All SE+ horses showed a positive response to both WBE and saliva of *Culicoides* (Fig. 1). The WBE of *C. nubeculosus* induced (1+) and (2+) reactions and *C. sonorensis* induced from (1+) to (3+) reactions. Responses to the saliva of both species ranged from (2+) to (4+). Control horses showed no reactivity to saliva, but two horses did respond (1+) to the WBE of both *Culicoides* species. Both the 2×2 and 2×4 comparisons revealed highly significant differences for the mean number of positive reactions for all preparations.

The amount of released histamine decreased with decreasing concentrations of the WBE of both insect species (Fig. 2). However, the histamine release in

Table 3
Number of responses to IDT at 24 h after the challenge for SE affected horses ($n = 9$) and control animals ($n = 9$)

Preparation	Concentration (μg/ml)	Skin reaction for horses with SE		Skin reaction for control horses	
		Negative	Positive	Negative	Positive
C. nubeculosus WBE[a]	5	5	4	8	1
	0.5	8	1	8	1
C. sonorensis WBE	5	4	5	7	2
	0.5	8	1	6	3
C. nubeculosus saliva	5	3	6	6	3
	0.5	8	1	4	5
C. sonorensis saliva	5	7	2	7	2
	0.5	9	0	9	0

[a] WBE, whole body extract.

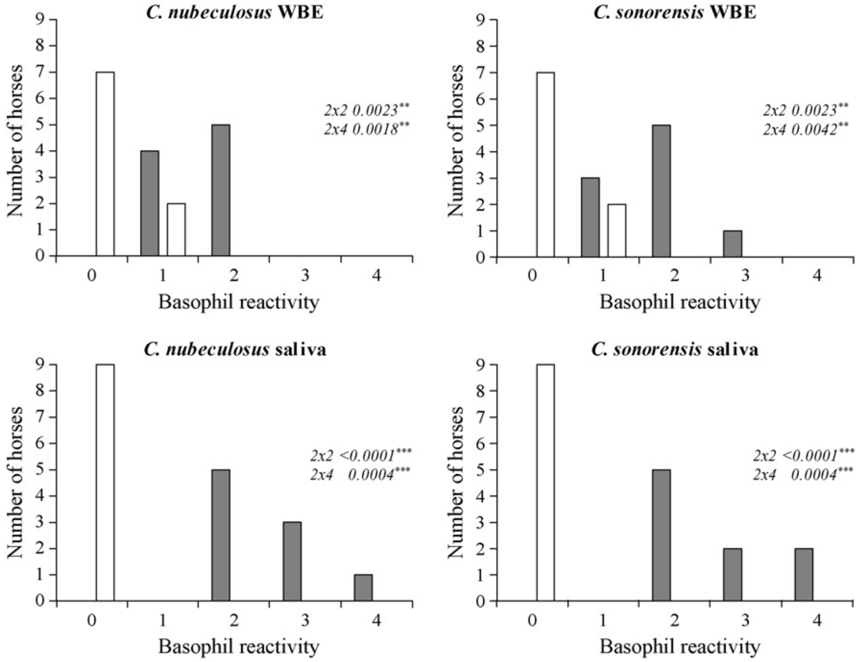

Fig. 1. Histamine release test results of SE affected horses (■, $n = 9$) and control animals (□, $n = 9$) to *Culicoides* whole body extracts (WBE) and saliva. The basophil reactivity indicates the total of allergen dilutions capable of inducing a histamine release. The number of responders is shown on the *Y*-axis. The italic numbers characterize the level of significance for 2×2 comparisons and 2×4 comparisons for the two groups of horses: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

response to the highest saliva concentration (5 μg/ml) in 7 of 9 SE+ horses was less than that induced by the second highest concentration (0.5 μg/ml).

3.3. ELISA

Culicoides-specific IgE was detected in the native serum of 31% of SE+ horses and in 0% of the control animals (Table 4). The majority of positive reactions were seen with the WBE of the two *Culicoides* spp. when compared with the saliva. *Culicoides*-specific IgG was found in 11% of the allergic and in 0% of the non-allergic horses. A slightly higher number of positive reactions was obtained for the WBE and saliva of *C. nubeculosus* than for the preparations of *C. sonorensis*.

The use of purified serum IgE and IgG greatly increased the number of positive reactions in SE+ horses (Fig. 3) whereas the reactivity of the control horses with the preparations remained negative.

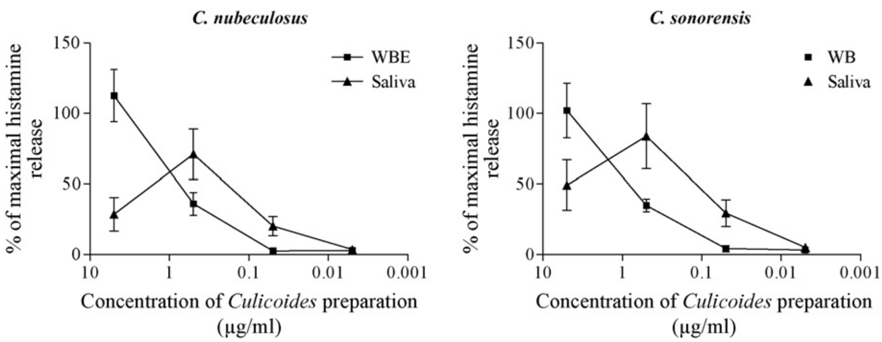

Fig. 2. Induction of a high dose hook effect in the histamine release test. The histamine release obtained for different dilutions of whole body extracts (WBE) and saliva of *C. nubeculosus* and *C. sonorensis* is shown for five SE+ horses. The release induced by allergen preparations is calculated as the percentage of maximal histamine release obtained after boiling the blood cells for 10 min. Results are expressed as means with S.E.M. indicated by horizontal bars.

Table 4
ELISA results for SE affected horses ($n = 9$) and control animals ($n = 9$)

Preparation	Immunoglobulin	ELISA reaction for horses with SE		ELISA reaction for control horses	
		Negative[a]	Positive[b]	Negative	Positive
C. nubeculosus WBE[c]	IgE	5 (1/4)	4 (4/0)	9 (9/0)	0
	IgG	8 (7/1)	1 (1/0)	9 (9/0)	0
C. sonorensis WBE	IgE	6 (5/1)	3 (3/0)	9 (9/0)	0
	IgG	9 (6/3)	0	9 (9/0)	0
C. nubeculosus saliva	IgE	7 (5/2)	2 (0/2)	9 (8/1)	0
	IgG	6 (4/2)	3 (3/0)	9 (8/1)	0
C. sonorensis saliva	IgE	7 (7/0)	2 (2/0)	9 (8/1)	0
	IgG	9 (7/2)	0	9 (7/2)	0

[a] Total negative (negative/borderline).
[b] Total positive (positive/strong positive).
[c] WBE, whole body extract.

3.4. Sensitivity, specificity, positive and negative predictive value

The results obtained for the three test systems are summarized in Table 4. For the evaluation of the IDT the concentrations with the lowest *P* values were chosen. At 30 min and 24 h p.i. a concentration of 5 μg/ml of all preparations was used to calculate sensitivity, specificity, PPV and NPV. At 4 h p.i., 0.5 μg/ml WBE and 0.05 μg/ml saliva concentrations of both *Culicoides* species were used.

4. Discussion

Intradermal testing with the highest concentration of the *Culicoides* preparations revealed that horses with SE had a significantly greater mean number of positive reactions at 30 min p.i. (immediate reaction) than non-allergic horses (Table 1). Some SE+ horses in this study failed to react to one or more of the intradermally injected *Culicoides* preparations. It has been reported previously that horses mildly affected by SE provoke false negative results in IDT (Halldorsdottir et al., 1989). One horse in this study did not show a response to any of the preparations, a second horse reacted exclusively and only weakly to the WBE of *C. nubeculosus*. Both animals displayed very mild symptoms of the allergy when compared to the other SE+ horses. Two additional horses failed to react to individual preparations while showing a strong response to the majority of the injected allergens. Recently, it has been demonstrated by comparative injections of allergen preparations into both sides of the cervical region of the same horse that mast cells are unevenly distributed in the skin (Hampel, 2007). Therefore, it is possible that the number of mast cells in certain areas used for IDT is not always representative for the allergic status of a horse resulting in false negative reactions to single allergen preparations.

At 4 h p.i. several control horses reacted to the *Culicoides* preparations (Table 2), but failed to show an immediate response at 30 min. This is similar to what

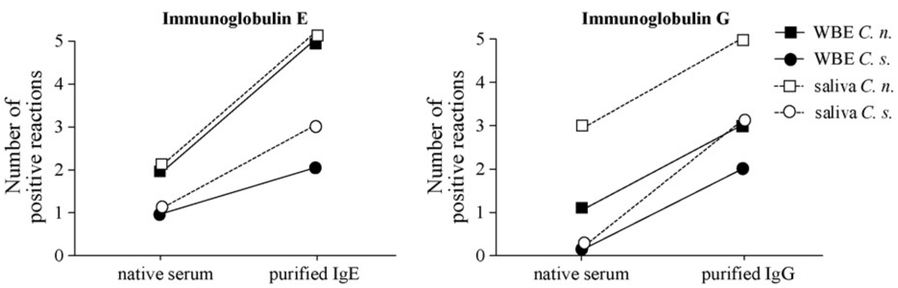

Fig. 3. Improved detection of *Culicoides*-specific IgE and IgG in the ELISA by purified serum immunoglobulins. Immunoglobulin E and G were affinity-purified from five SE+ horses. *Culicoides*-specific antibodies were determined by ELISA using the whole body extract (WBE) and saliva of *C. nubeculosus* (*C.n.*) and the WBE and saliva of *C. sonorensis* (*C.s.*). Positive reactions for IgE and IgG are shown as comparison of the native serum versus the purified immunoglobulins.

has been reported previously where non-allergic horses responded to a variety of allergen preparations at 4 h p.i. in the absence of an early reaction (Lorch et al., 2001). An underlying cause may be the presence of irritant substances in the preparations that cause a non-allergen mediated inflammation. Such proinflammatory and vasoactive components have been detected in salivary gland extracts as well as in the saliva of *Culicoides* spp. and have been found capable of inducing edema and erythema when applied intradermally to non-allergic laboratory animals and humans (Perez de Leon et al., 1997; Langner et al., 2007).

At 24 h p.i. horses with and without SE showed positive skin reactions to the *Culicoides* preparations. However, with exception of *C. sonorensis* saliva, the number of responders was higher in the group of allergic horses indicating a delayed reaction and the involvement of allergen-specific T-cells (Halldorsdottir et al., 1989; Fadok and Greiner, 1990).

The HRT revealed positive results for the highest concentration of the *Culicoides* WBE in all SE+ horses, but also for two non-allergic horses. The false positive results may be explained by a subclinical sensitization of individual control horses as previously discussed for non-allergic dogs in flea-rich environments (Kunkle et al., 2000; Laffort-Dassot et al., 2004). In contrast, none of the control horses reacted with the saliva of the two *Culicoides* species. Therefore, it remains possible that the obtained false positive results for the WBE are non-specific reactions. Whole body extracts, unlike the saliva consisting of numerous ingredients, may contain components that are capable of inducing a non-allergen related basophil degranulation in individual horses. Basophils carry a repertoire of antibodies on their surface specific for various antigens (Marti et al., 1999; Geiben, 2003) that may bind structurally similar proteins of the *Culicoides* WBE. In addition, carbohydrates present in the WBE may cross-link the highly glycosylated surface-bound antibodies resulting in a non-specific basophil cell activation and degranulation as described previously (Pramod et al., 2007).

In most of the allergic horses, the highest concentration of the saliva induced little or no histamine release as compared to the 10-fold lower concentration of the saliva. This poor dilutional linearity, also known as a prozone phenomenon or high dose hook effect, has been observed for various serological tests as well as for passive immunizations and is caused by suboptimal antigen–antibody relations (Taborda and Casadevall, 2001; Nnoruka and Ezeoke, 2005; Akamatsu et al., 2006). To our knowledge, this phenomenon has not yet been described for a cell-based allergy test. Studies on agglutination tests have shown that an overdose of agglutinating antibodies leads to the binding of single erythrocytes and a reduced or missing cross-linkage of cells (Guven et al., 2006). Similarly, extensive amounts of allergens may predominantly lead to the occupation of individual surface-bound antibodies on the basophils rather than the cross-linkage of multiple antibodies that is required for the cell activation. Overall, our findings indicate that dilution series of allergen preparations are necessary to avoid false negative results using the HRT or similar systems.

The utilization of ELISA systems for the diagnosis of allergies is based on the hypothesis that atopic individuals display higher titers of allergen-specific antibodies than non-atopic individuals (Portengen et al., 2004; Mimura et al., 2004). Previously, an ELISA based on epsilon chain antigenic peptides showed a positive reaction for 24 of 64 atopic horses to different allergen preparations including grass, grainmill dust, mosquito and horsefly (Kalina et al., 2003). A second study utilizing a recombinant human FcεRIα chain or polyclonal antisera to equine IgE showed low percentages (15 and 11%, respectively) of false positive reactions to different allergen preparations, but high percentages (63 and 89%, respectively) of false negative reactions (Lorch et al., 2001). Similar results were obtained in this study when native serum was used for the detection of *Culicoides*-specific IgE and IgG (Table 5). However, the use of purified serum immunoglobulins showed an improved detection of both insect-specific IgE and IgG in SE+ horses (Fig. 3). These findings indicate that titers of *Culicoides*-specific antibodies may be below the detection limit when native serum is used. The majority of IgE is effectively bound to the surface of mast cells and basophils via the high affinity FcεRIα resulting in a short half-life of IgE in the blood stream of 2.5 days and low serum concentrations ranging between 0.4 and 108.9 μg/ml in horses (Hirano et al., 1983; McAleese and Miller, 2003; Wagner et al., 2003; Wilson et al., 2006). By contrast, the equine genome displays genes for seven different IgG isotypes (Wagner et al., 2004) and it remains unclear as to which of them are involved in immediate hypersensitivities in horses. Thus, no finally convincing data are available that describe the titers and kinetics of allergen-specific IgG in horses. The purification of IgE and IgG may not only have increased the concentration of *Culicoides*-specific antibodies, but also excluded the competition for the *Culicoides* proteins. Both isotypes were shown to bind to *Culicoides* salivary gland tissue (Wilson et al., 2001; Hellberg et al., 2006). Finally, the improved detection of *Culicoides* components by purified IgG

Table 5
Calculation of sensitivity, specificity, positive predictive value and negative predictive value for IDT, HRT and ELISA

Method	Preparation		Sensitivity	Specificity	PPV[a]	NPV[b]
Intradermal test 30 min/4 h/24 h	WBE[c]	*C.n.*[d]	0.78/0.78/0.44	1.00/0.89/0.89	1.00/0.88/0.80	0.82/0.80/0.62
		C.s.[e]	0.78/0.78/0.56	1.00/0.67/0.78	1.00/0.70/0.71	0.82/0.75/0.64
	Saliva	*C.n.*	0.78/0.89/0.67	1.00/1.00/0.67	1.00/1.00/0.67	0.82/0.75/0.67
		C.s.	0.56/0.78/0.22	1.00/1.00/0.78	1.00/1.00/0.50	0.69/0.75/0.78
Histamine release test	WBE	*C.n.*	1.00	0.78	0.82	1.00
		C.s.	1.00	0.78	0.82	1.00
	Saliva	*C.n.*	1.00	1.00	1.00	1.00
		C.s.	1.00	1.00	1.00	1.00
ELISA IgE/IgG	WBE	*C.n.*	0.44/0.11	1.00/1.00	1.00/1.00	0.64/0.53
		C.s.	0.33/ns[f]	1.00/ns	1.00/ns	0.6/ns
	Saliva	*C.n.*	0.22/0.33	1.00/1.00	1.00/1.00	0.56/0.6
		C.s.	0.22/ns	1.00/ns	1.00/ns	0.6/ns

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.
[c] WBE, whole body extract.
[d] *C.n.*, *Culicoides nubeculosus*.
[e] *C.s.*, *Culicoides sonorensis*.
[f] ns, no statistical analysis as allergen is constant.

may be due to an enrichment of certain IgG subclasses by protein G (predominantly IgG1 and IgG4, unpublished data).

5. Conclusions

Comparison of the three allergy tests revealed a considerably higher reliability for the HRT and IDT (immediate and late-phase reaction) than for the ELISA (Table 5). The IDT, as well as the HRT, are biological assays that mimic the natural hypersensitivity reaction mediated by sensitizing antibodies. The highest reliability for the IDT was found at 30 min p.i. for all *Culicoides* preparations and at 4 h p.i. for the saliva. Unlike the HRT, the IDT reactions result from not only the insect specific sensitization of the effector cells, but also non-specific inflammatory reactions at the administration site. These reactions occurred simultaneously with the late-phase (4 h p.i.) and delayed (24 h p.i.) response of the allergic reaction and partly hindered the discrimination between horses with and without SE. The HRT is a less invasive method than IDT and displayed at the same time a similar or higher reliability, but exclusively recorded the immediate allergic reaction. The ELISA determines the level of circulating immunoglobulin in the serum which may not reflect the tissue-fixed antibodies and their role in the allergic reaction. The use of purified IgE and IgG revealed evidence that SE+ horses may have higher levels of *Culicoides*-specific antibodies than the control animals but titers in the native serum were below the detection limit of the ELISA. The comparison of the two *Culicoides* species showed no significant differences for the IDT and HRT indicating the presence of species-shared allergens in both the native and exotic midges. By contrast, the determination of *Culicoides*-specific IgG by ELISA revealed a higher number of positive reactions for the native *C. nubeculosus* when compared with *C. sonorensis*, suggesting the possibility of additional target proteins in the native species and/or differences in affinity and titer of antibodies with specificity for common proteins. The use of saliva versus the WBE of the insects greatly increased the specificity of the HRT and also improved both the sensitivity and specificity of the late-phase reaction of the IDT. This suggests that clean allergen preparations can reduce false positive results as well as false negative results. Overall, the results of this study clearly indicate that cell-based allergy tests are more reliable for the diagnosis of SE in horses than serological tests. However, a careful evaluation of the horse's clinical signs and medical history combined with *in vivo* or *in vitro* testing is necessary for an accurate diagnosis of the disease.

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